THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

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NEW YORK, N. Y. 10022
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Application for Research Grant
(Use extra pages as needed)

Date: June 27, 1975

1. Principal Investigator (give title and degrees):
Koji Yoshinaga, Ph.D.
Associate Professor of Anatomy

2. Institution & address:
Laboratory of Human Reproduction and Reproductive Biology
Harvard Medical School
45 Shattuck Street, Boston, Massachusetts 02115

3. Department(s) where research will be done or collaboration provided:
Laboratory of Human Reproduction and Reproductive Biology

4. Short title of study:
Effects of nicotine on pregnancy.

5. Proposed starting date: January 1, 1976
6. Estimated time to complete: 3 years
7. Brief description of specific research aims: Nicotine has been reported to exert deleterious effects on pregnancy. Nicotine acts not only on the genital tract to alter its movement, but also on the pituitary gland to inhibit the secretion of luteinizing hormone and prolactin. Since these two hormones play important roles in stimulating ovarian hormone secretion, the deleterious effects of nicotine on pregnancy may be through the hypothalamo-pituitary-ovarian axis. The aims of the proposed research are to determine if nicotine exerts direct action on the genital tract-embryo and/or indirect action on the endocrine system and to clarify the mode of action of nicotine on pregnancy with particular emphasis on hormone imbalance in the hypothalamo-pituitary-ovarian axis caused by nicotine.

8. Brief statement of working hypothesis:

Inhibitory action of nicotine on the secretion of luteinizing hormone may be mediated by inhibition of release and/or production of gonadotrophin releasing hormone in the hypothalamus. Suppression of the secretion of luteinizing hormone and prolactin by nicotine will result in subnormal secretion of progesterone and estrogen by the ovary and normal progress of pregnancy will be interfered. If nicotine acts mainly on the endocrine system, supplement of nicotine treated-animals with ovarian hormones will overcome the deleterious effects of nicotine on pregnancy. If they are not overcome, direct effects of nicotine on the genital tract and/or embryo will become obvious.

9. Details of experimental design and procedures (append extra pages as necessary)

Introduction

Although cigarette smoking has been reported to exert deleterious effects on pregnancy (1,2), few analytical studies have been done on the mode of action of inhaled substances. It has not been determined whether the deleterious effects of nicotine on pregnancy (3) are direct on the genital tract-embryo or indirect on the endocrine system which regulates the reproductive processes (the hypothalamo-pituitary-ovarian axis).

In early pregnancy movement of cilia and muscle of the genital tract facilitates transport of fertilized ova through the Fallopian tube and location of ova in implantation sites of the uterus. It has been shown that the cilial movement of the Fallopian tube and muscle contraction of of the Fallopian tube and the uterus are influenced by ovarian hormones, estrogen and progesterone (4,5). Although nicotine alters the contractile activity of the genital tract, the effect of nicotine is still influenced by estrogen and progesterone (6). If a small amount of estrogen is administered to pregnant mice while fertilized ova are in the Fallopian tube, transport of the ova is blocked and the ova are retained in the Fallopian tube for a prolonged period of time (7). Since nicotine affects contracti activity of the Fallopian tube (6), nicotine may alter the speed of ovum

transport resulting in its untimely arrival into the uterus. We have much evidence that asynchrony of the ovum arrival and preparation of the uterus to receive the ovum often result in unsuccessful implantation of the ovum (8,9,10). Prolonged gestation period in nicotine treated rats observed by Becker et al. (3) may be due to a delay in ovum implantation: this phenomenon is frequently seen in the rat whose estrogen secretion is hindered by agents such as tranquilizers(11) or reserpine (12) and under the condition of concurrent lactation(8) and stress (13).

It has been shown that nicotine delays and suppresses the secretion of luteinizing hormone (14) and prolactin (15). Since these two hormones stimulate, with various combinations, progesterone secretion (18,19,20), suppression of luteinizing hormone and prolactin will reduce progesterone secretion (19, 21). Luteinizing hormone has also been shown to stimulate estrogen secretion (22). When luteinizing hormone level is lowered by neutralizing with its antibody, estrogen secretion is supppressed (23).

A study on placental transfer and distribution of nicotine in the fetus shows that nicotine concentration in fetal circulation is higher and its clearance rate slower than those in the mother (24). Thus direct effect of nicotine on the embryo cannot be eliminated.

In this proposed research we aim to analyze the effects of nicotine on pregnancy by determining which hormones in the hypothalamo-pituitary-ovarian axis are suppressed by nicotine. The obtained results will be correlated with deleterious effects of nicotine on other biological parameters. During the first year we will concentrate our effort on the period between ovulation and ovum implantation. Studies on later stages of pregnancy will be carried out during the subsequent years.

Experimental design

The rat will be used in this study as an experimental animal. Pregnant rats treated with various doses of nicotine will be sacrificed (6 rats in a group) at 3 hour intervals from day 0 to day 6 of pregnancy to collect samples for measurement of hormones and other biological parameters. The hormones to be measured are: gonadotrophin releasing hormone (also called as luteinizing hormone releasing hormone)in the hypothalamus; luteinizing hormone, follicle stimulating hormone and prolactin in the pituitary gland and serum; and ovarian steroid hormones in the serum (progesterone, 20α-hydroxypregn-4-en-3-one, estradiol and estrone).

Other biological parameters are: location, appearance and viability of ova; number of implantation sites in the uterus; number and weight of corpora lutea; and time of ovum implantation. By locating the ova in the genital tract at various stages of pregnancy, we can estimate the speed of ovum transport. Appearance of ova (the size and number of blastomeres) will reveal their developmental stages or degree of degeneration. The number of corpora lutea will be considered as the number of ova ovulated. This number will be used for calculation of the percentage of ova developed to various stages of embryonic development. Viability of ova will be examined by determining their ability to develop after transfer into the uterus of recipients (pseudopregnant rats).

The mode of action of nicotine will be deduced from comparison of the secretory pattern of hormones with other biological parameters. The obtained conclusions will be tested by determining if compensation of the reduced hormone will overcome the nicotine effect. If the hormone therapy does not overcome the nicotine effect, nicotine is considered to have exerted direct effect on the genital tract and/or embryo.

Procedure

1. Animals

Young adult female rats (60 days old) will be purchased from Charles River Breeding Laboratories, Wilmington, Mass.. The estrous cycles will be traced by vaginal smear method and the rats at proestrous stage will be placed with fertile males overnight. The vaginal smear will be examined in the following morning; those rats with spermatozoa are considered pregnant and this day will be designated as day 1 of pregnancy.

2. Treatment of rats with nicotine

From day 0 (the day of proestrus) the rats will be injected subcutaneously twice daily (at 900 hr and 1800 hr) with high, intermediate or low dose of nicotine (5, 1 or 0.2 mg/ day; namely 15, 3 or 0.6 mg nicotine tartrate) disolved in saline. Control rats will receive the vehicle only (0.9% NaCl).

3. Collection of samples

The rats will be sacrificed by decapitation at 3 hour intervals from 900 hr on day 0 till 900 hr on day 6 of pregnancy. Implantation of the ovum normally takes place in the afternoon of day 5.

Immediately after decapitation blood will be collected from neck blood vessels. Serum will be separated by centrifugation after clot formation. The hypothalamus and pituitary gland will be collected from the head and will be frozen on dry ice as soon as possible. The ovaries, Fallopian tubes and uterus will be dissected out. The Fallopian tubes and the uterus will be flushed with saline for collection of ova according to the method previously reported (25) and to that of Dickmann (26). The number of ova and their size and appearance

(developmental stage or degree of degeneration) will be recorded. The numbers of corpora lutea and implantation sites are also recorded. The sites of ovum implantation at very early stages (afternoon of day 5) will be visualized by a blue dye injection (27).

4. Measurement of hormones

A. Gonadotrophin releasing hormone (GnRH).

A radioimmunoassay method for GnRH reported in our earlier publications (28,29) will be used. The hypothalamic area which was cut out immediately after decapitation and kept frozen will be homogenized in 1 ml 0.2 M ice chilled acetic acid. The homogenate will be stored for 24 hr at 4 C and cenrifuged at 20,000 Xg for 1 hr. Duplicate aliquots of 200 µl supernatant will be neutralized with 200 µl 0.2M NH_4OH. After addition of 400 µl 0.2M tris-acetate buffer (pH 7.3), 100 µl antiserum to GnRH (1:300-1:1,000), and 100 µl ^{125}I-GnRH, the mixture will be incubated for 4 hr at room temperature and subsequently 44 hr at 4C. After incubation antigen-antibody complex will be separated by precipitation of unreacted labeled GnRH with dextran T70-coated charcoal. The supernatant will be subjected for counting.

B.Pituitary hormones: luteinizing hormone(LH), follicle stimulating hormone (FSH) and prolactin.

LH, FSH and prolactin will be measured by radioimmunoassay using NIAMDD (National Institute of Arthritis and Metabolic and Digestive Diseases) kits. The pituitary will be homogenated in phosphate buffered saline. The tissue concentration in the homogenate will be 10 mg wet weight/ml. The extract will be assayed for LH, FSH and prolactin. These hormones in the serum will be likewise assayed.

C. Ovarian steroid hormones:

Steroids in serum will be extracted with ether and progesterone, 20α-hydroxypregn-4-en-3-one(20α-OH-P), estradiol and estrone will be separated by Sephadex LH-20 column chromatography. 20α-OH-P will be converted to progesterone by chromic acid oxidation. In our study the values obtained for 20α-OH-P by radioimmunoassay were within the comparable range of those measured by gas liquid chromatography(19).

5. Viability test of ova

In order to examine if nicotine acts directly on the ovum and affects its later development, ova will be collected from the Fallopian tube or the uterus of nicotine treated rats and transferred to untreated recepients according to the method described in an earlier publication (25). Pseudopregnant rats will be used as the recipients of the ova. Pseudopregnancy will be induced by mechanical stimulation of the uterine cervix on the day of estrus(the last day of vaginal cornification is designated as day 1 of pseudopregnancy). The day of pseudopregnancy and the age of the ova will be synchronous.After transfer of the ova the recipients will be laparotomized on days 9, 14 and 19 of(pseudo-)pregnancy and number of implantation sites,and developing fetuses will be recorded.

6. Hormone therapy of the nicotine treated rats

From the data obtained from hormone measurement and other biological parameters we will know which hormone is suppressed by nicotine at what stage of pregnancy. Since suppression of hormones at higher levels (hypothalamus and pituitary) will be reflected by suppression of ovarian steroids, nicotine treatment is expected to result in a reduction of estrogen or progesterone. In order to find out if

supplement of reduced hormone(s) will overcome the nicotine effect, estrogen and/or progesterone will be administered to nicotine treated rats and subsequent embryonic development will be studied. The dose of hormones and period of treatment will be decided after obtaining the data on hormone levels.

References

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

This research will be conducted at the Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, 45 Shattuck Street, Boston, Massachusetts. We have enough laboratory space (1 x 460 sq. ft; 2 x 230 sq ft) and office space to conduct this research. Besides these spaces we share one instrument room where 3 refrigerated centrifuges, 1 ultracentrifuge, 1 scintillation counter, 1 gamma counter, 1 lyophilizer, 1 gas liquid chromatograph and 1 spectrophotometer are available for us to use. We also have sufficient space and cages to house up to 360 rats at one time, which is more than adequate for the proposed research. Other items of major equipment are fraction collectors, pH meters, balances, microscopes, electrophoretic apparatus, ovens, freezers and refrigerators.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

12. Biographical sketches

Yoshinaga, Koji

Title: Ph.D., Associate Professor of Anatomy

Born: REDACTED Sex: Male

Nationality: REDACTED

Education:

		Scientific Field
B.S.	Univ. of Tokyo, Japan	Animal Physiology
M.D.	" " " "	" "
REDACTED Ph.D.	" " " "	" "
	Worcester Fdn. Exp. Biol. Shrewsbury, Mass. Postdoctoral Training	Reprod. Physiol

Honors: Awardee, Population Council Fellowship 1962-63, 1964-65.

Awardee, Lalor Found. Fellowship 1965-1966 for study at Cambridge University, Cambridge, England.

Major Research Interest: Endocrinology of female reproduction

Role in Proposed Project: Principal Investigator

Research and/or Professional Experience:

Associate Professor of Anatomy (full-time) Harvard Medical School, Boston, Mass. 7/1/72 - present.

Research, supervision of postdoctoral fellows and teaching histology laboratory to medical and dental students at Harvard Medical School. Research projects: Ovo-implantation and ovarian function.

Assistant Professor of Anatomy (full-time) Harvard Medical School, Boston, Mass. 7/1/69 - 6/30/72.

Research and teaching activities, same as above.

Research Associate in Anatomy (full-time) Harvard Medical School, Boston, Mass. 2/1/69 - 6/30/69.

Research on the same projects.

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Staff Scientist (full-time), The Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 12/1/66 - 1/31/69.

Research on female reproduction. Projects: estrogen secretion by the rat ovary, uterine sensitivity and ovo-implantation.

Teaching staff of the Training Program in the Physiology of Reproduction for postdoctoral fellows.

Visiting Scientist (full-time), Agricultural Research Council, Unit of Reproductive Physiology & Biochemistry, University of Cambridge, Cambridge, England. 11/1/64 - 11/30/66.

Research Projects: Hormonal requirement for ovo-implantation, steroid hormone determination in the ovarian venous blood in the rat.

Staff Scientist (full-time), The Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 1/15/63 - 10/31/64.

Research Project: Stimulatory effect of 3'5'-cyclic AMP and analogues on the synthesis of protein and phospholipids in the rat uterus.

Trainee in the Training Program in the Physiology of Reproduction (full-time), The Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 1/15/61 - 1/14/63.

Training in the physiology of reproduction in general, local action of estrogen on the uterus.

Research Fellow (full-time), University of Tokyo, Tokyo, Japan. 4/1/60 - 12/31/60.
Research Project: Delayed implantation in lactating rats. Parturition of superfetation rats.

13. Five publications pertinent to the proposed work;

1) Yoshinaga, K. and C.E. Adams (1966) Endocrine aspects of egg implantation in the rat. J. Reprod. Fert. 12, 583.
(relevant to egg transfer technique).

2) Yoshinaga, K. and R.O. Greep (1971) Local inhibition of ovo-implantation in the rat. Endocrinology 88, 627.
(describes ovarian hormone regulation of uterine receptivity for ovum implantation)

3) Yoshinaga, K. and J.J. Ford (1974) Luteotrophic complex in lactating rats. In "Gonadotropins and gonadal function" Editor:N.R. Moudgal, Acad. Press, New York. p.260.
(gonadotrophin control of ovarian steroid secretion)

4) J.J. Ford and K. Yoshinaga (1975) The role of prolactin in the luteotrophic process of lactating rats. Endocrinology 96, 335.
(drug effects on LH and prolactin and ovarian progestin secretion)

5) M. Takahashi, J.J. Ford, K. Yoshinaga and R.O. Greep (1975) Effects of cervical stimulation and anti-LH releasing hormone serum on LH releasing hormone content in the hypothalamus. Endocrinology 96, 453.
(method for GnRH measurement in the hypothalamus; also describes measurement of LH and ovarian steroids)

4.

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Koji Yoshinaga (includes 16% fringe benefits)	40%	REDACTED
Technical laboratory technician to be recruited	100%	REDACTED
Secretary Stella Nieland (includes 14.5% fringe benefits)	25%	
	Sub-Total for A	REDACTED

B. Consumable supplies (by major categories)

Rats, purchase & maintenance	1,300
Isotopes and chemicals	1,500
Glassware and plastic disposables for hormone assay	1,000
Maintenance service contract for scintillation counter	500
Sub-Total for B	4,300

C. Other expenses (itemize)

Travel to scientific meetings	600
Publication costs	500
Sub-Total for C	1,100
Running Total of A + B + C	28,800

D. Permanent equipment (itemize)

Sub-Total for D	0
E	4,320
Total request	$ 33,120

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R	$4,500	$1,200	0	$4,575	$35,075
Year 3		4,500	1,300	0	4,815	36,915

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Role of feto-placental unit in maintenance of pregnancy	NIH-HD-06467	$114,439	2-1-72 - 7-31/75
Decidual tissue as an endocrine gland	Milton Fund, Harvard University	2,800	7-1-75 - 6-30-76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Endocrine regulation of ovum implantation	NIH HD-09006	(for 3 yr period) $187,742	9-1-75 - 8-31-78
Luteotrophic complex	NSF BM-75-19998	213,291	9-1-75 - 8-31-78
Role of decidual tissue in pregnancy	NIH	183,563	1-1-76 -12-31-78

Budget information: If other NIH and NSF grants are fully funded, % time of the principal investigator will be reduced from 40% to 20%. If the Council for Tobacco Research would agree, the 20% will be used for partial support of a post-doctoral fellow in order to keep up pace of the work.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Koji Yoshinaga

Signature Koji Yoshinaga Date 6/27/75

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Responsible officer of institution

Typed Name Henry C. Meadow

Title Executive Secretary, Committee for Research and Development

Signature Henry C. Meadow Date

Telephone 617 (Area Code) 734-3300 (Number) 441 (Extension)

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